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Decoy Oligodeoxynucleotide Characterization of Transcription Factors Controlling Endothelin-B Receptor Expression in Vascular Smooth Muscle Cells

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ABSTRACT

Endothelin-1 is not only a powerful vasoconstrictor but also a potent mitogen for vascular smooth muscle cells (SMC), acting through both the endothelin-A and endothelin-B receptor (ET_B-R). Although vascular SMC are known to express the ET_B-R, its transcriptional regulation has not been studied thus far. Here we demonstrate that the potent inhibitor of nuclear factor κB activation, pyrrolidine dithiocarbamate (PDTC; 30–100 μM), induces de novo ET_B-R expression in rat aortic and mesenteric cultured SMC. Electrophoretic mobility shift analyses revealed that besides inhibition of nuclear factor κB, PDTC enhances activator protein-1 (AP-1), CCAAT/enhancer-binding protein (C/EBP), and GATA-2 activity in these cells. Preincubation of PDTC-stimulated cells with appropriate decoy oligodeoxynucleotides confirmed the involvement of these three transcription factors, namely that of AP-1, in ET_B-R expression. The stimulatory effect of PDTC on ET_B-R expression was also confirmed functionally by monitoring an enhanced ET-1-induced apoptosis in PDTC-treated cells that was sensitive to the ET_B-R antagonist, BQ788. Taken together, these findings demonstrate that C/EBP, GATA-2, and in particular AP-1 can control ET_B-R expression in vascular SMC. They further support the notion that ET_B-R expression in these cells may play an important role in cardiovascular complications, such as restenosis following angioplasty that in the early phase is characterized by prominent SMC apoptosis.

The endothelins constitute a family of three closely related peptides (ET-1, ET-2, and ET-3) with diverse physiological and pathophysiological actions ranging from embryogenesis to heart failure. In the cardiovascular system, ET-1 is the main representative of these 21 amino acid peptides. Predominantly formed by endothelial cells lining the luminal surface of the blood vessels, ET-1 is not only a powerful vasoconstrictor, but also a potent mitogen for vascular smooth muscle cells (SMC). It is derived from a 212 amino acid precursor, preproendothelin-1, that is sequentially processed to big ET-1 and ET-1 by a furin-like protease and one or several endothelin-converting enzymes (Parris and Webb, 1997; Schiffrin et al., 1997). ET-1 exerts its biological effects mainly through activation of two types of seven transmembrane-spanning G protein-coupled receptors, designated ET_A-R and ET_B-R. Although SMC express both types of receptors, ET_A-R activation appears to predominantly modulate SMC tone and proliferation in arteries, whereas in veins ET-1 seems to exert these effects primarily through activation of the ET_B-R (Douglas and Ohlstein, 1997). Endothelial cells also express an ET_B-R, the activation of which promotes the release of nitric oxide and prostacyclin, thereby potentially limiting an excessive ET_A-R and/or ET_B-R-mediated, ET-1-induced vasoconstriction.

The development of specific endothelin receptor antagonists has provided some important insights into the pathophysiological significance of the endothelin system in cardiovascular disease (Kirchengast and Munter, 1999). Thus,

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ABBREVIATIONS: ET-1, -2, -3, endothelin-1, -2, -3; SMC, smooth muscle cells; raSMC, rat aortic cultured SMC; AP-1, activator protein-1; PDTC, pyrrolidine dithiocarbamate; ET_A-R, endothelin-A receptor; ET_B-R, endothelin-B receptor; PTCA, percutaneous transluminal coronary angioplasty; ODN, oligodeoxynucleotide(s); dODN, decoy ODN; EMSA, electrophoretic mobility shift analysis; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; EF-2, elongation factor 2; bp, base pair(s); VCAM-1, vascular cell adhesion molecule-1; iNOS, inducible nitric-oxide synthase; C/EBP, CCAAT/enhancer-binding protein; NF-κB, nuclear factor κB; PMA, phorbol 12-myristate 13-acetate; TNFα, tumor necrosis factor α; IL-1β, interleukin-1β; IFNγ, interferon-γ.

ET-1 may contribute to the intimal hyperplasia of venous bypass grafts through activation of the ET_A-R (Dashwood et al., 1998) and/or ET_B-R (Porter et al., 1998). Moreover, an increased synthesis of ET-1 (Wang et al., 1996) with consecutive activation of the ET_A-R and/or ET_B-R (Douglas et al., 1995; Tsujino et al., 1995) has been implicated in restenosis following percutaneous transluminal coronary angioplasty (PTCA). Unfortunately, most of the endothelin receptor antagonists developed thus far seem to encounter toxicological problems when administered to patients over a prolonged period of time. Therefore, elucidating the transcriptional mechanisms involved in endothelin receptor expression may provide a novel approach to suppress the activity of the endothelin system in the aforementioned cardiovascular complications.

Recent progress in molecular biology has spurred the development of new techniques for specifically inhibiting expression of a target gene. Antisense oligodeoxynucleotides (ODN) are single-stranded synthetic DNA molecules that after internalization, a process which is dependent on the sequence of the ODN (Etore et al., 1998), hybridize with the expressed mRNA of the target gene, hence preventing its translation (Phillips and Gyurko, 1997). A related powerful tool to prevent transcription of a target gene is the transfection of cells with double-stranded phosphorothioate-stabilized decoy ODN (Morishita et al., 1998). After entering the cells, these decoy ODN scavenge endogenous transcription factors, thereby preventing them from binding to the promoter of the target gene and effectively suppressing mRNA synthesis. In addition, decoy ODN can be used to directly study the transcriptional control of a gene of interest in cultured cells as well as in native cells in situ. By employing this technique, for example, *cis*-elements regulating basal transcription of the ET_A-R gene in a rat SMC line have recently been identified (Yamashita et al., 1998).

In the present study we have used the decoy ODN technique in combination with electrophoretic mobility shift analysis (EMSA) to identify transcription factors involved in ET_B-R expression in cultured SMC from the aorta and mesenteric vascular bed of the rat.

Materials and Methods

Cell Culture. SMC were isolated from the aorta of male Wistar rats (250–300 g body weight) by using the explant technique essentially as described (Krzesz et al., 1999). They were seeded into collagen type 1 or gelatin-coated 60-mm Petri dishes for EMSA and Western blot analysis or into 6-well plates for RT-PCR analysis (2 mg/ml collagen or gelatin in 0.1 M HCl for 30 min at ambient temperature). All incubations were performed in Waymouth medium containing 10% fetal bovine serum (FBS; Life Technologies, Karlsruhe, Germany) with SMC grown to at least 80% confluence. Incubations were terminated by washing the cells with Hanks' balanced salt solution (HBSS) followed by mRNA or protein extraction. Cell viability was assessed by light microscopy as well as by a colorimetric assay with the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Denizot and Lang, 1986).

SMC from the mesenteric vascular bed of the rat were isolated after enzymic digestion for 1 h at 37°C with 10 mg/ml collagenase, 20 mg/ml BSA, 5 mg/ml elastase, 10 mg/ml trypsin inhibitor, and 10 mg/ml DNase followed by filtration of the cell suspension through a nylon membrane. The isolated mesenteric SMC were cultured in the same way as the aortic SMC described above.

Cells used for the experiments described below were derived from passages 2 to 4 of individual preparations (i.e., aorta and mesenteric vascular bed from different rats). The homogeneity of the cultured SMC was assessed by positive immunostaining for smooth muscle α-actin as described (Krzesz et al., 1999).

RT-PCR Analysis. Total RNA was isolated from the SMC by solid-phase extraction with the RNeasy kit from Qiagen (Hilden, Germany). Reverse transcription and polymerase chain reaction were performed essentially as described previously (Krzesz et al., 1999). Amplification of elongation factor 2 (EF-2) cDNA served as an internal standard. The following primers with the respective GenBank accession number, position of the PCR product in the coding sequence, and predicted size were used for amplification: ET_A-R (M60786, position 713-1258, 546 bp) 5′-TTCGTCATGGTACCCTTCGA-3′ (sense) and 5′-GATACTCGTTCCATTCATGG-3′ (antisense); ET_B-R (X57764, position 1216–1689, 474 bp) 5′-TTCACCTCAGCAGGATTCTG-3′ (sense) and 5′-AGGTGTGGAAAGTTAGAACG-3′ (antisense); vascular cell adhesion molecule-1 (VCAM-1; M84488, position 829-1933, 1105 bp) 5′-TGGAGCAAGAAATTAGATAATGG-3′ (sense) and 5′-CACATGTACAGGAGATGATGA-3′ (antisense); inducible nitric-oxide synthase (iNOS; D44591, position 22–848, 827 bp) 5′-ATGGCTTGCCCCTGGAAGTTTCTC-3′ (sense) and 5′-CCTCTGATGGTGCCATCGGGCATCTG-3′ (antisense); EF-2 (Z11692, position 1990–2207, 218 bp) 5′-GACATCACCAAGGGT-GTGCAG-3′ (sense) and 5′-GCGGTCAGCACACTGGCATA-3′ (antisense).

To ensure that the PCR amplification was indeed semiquantitative, i.e., in the linear phase of the exponential amplification curve, each PCR protocol was established for different numbers of cycles and amounts of cDNA. Furthermore, for each newly synthesized cDNA, the abundance of EF-2 cDNA was measured first, calculated, and used as a reference point to adjust the amount of cDNA from each sample for PCR amplification.

Western Blot Analysis. Analysis of ET_B-R protein expression in the cultured smooth muscle cells was performed as described (Hecker et al., 1994) with the modification that the whole cell homogenate was additionally treated for 15 min with 0.5% (w/v) CHAPS at 0–4°C to enrich the membrane-bound ET_B-R protein in the 10,000*g* supernatant. Protein extracts (10–50 μg of protein per lane) were separated by denaturing 10% polyacrylamide gel electrophoresis in the presence of SDS according to standard protocols and then transferred to a BioTrace polyvinylidene fluoride transfer membrane (Pall Corporation, Roßdorf, Germany). Transferred proteins were probed by an ET_B-R-specific antiserum (α-CLK23; Cramer et al., 1997) at a dilution of 1:2500. Visualization of the protein bands was achieved by using a secondary anti-rabbit antibody conjugated to horseradish peroxidase (1:3000 dilution; Sigma-Aldrich, St. Louis, MO), and the chemiluminescence detection method (SuperSignal chemiluminescent substrate; Pierce Chemical, Rockford, IL) was used followed by exposure to an autoradiography film Hyperfilm MP (Amersham Pharmacia Biotech, Buckinghamshire, England). Thereafter, the loading and transfer of equal amounts of protein in each line was verified by staining of the protein bands on the transfer membrane with India Ink followed by densitometry.

EMSA. Nuclear extracts from the cultured SMC were prepared as described previously (Krzesz et al., 1999). The double-stranded gel shift oligonucleotides (Santa Cruz Biotechnology, Heidelberg, Germany) for activator protein-1 (AP-1, 5′-CGCTTGATGACTCAGCCGGAA-3′); CCAAT/enhancer-binding protein (C/EBP, 5′-TGCAGATTGCGCAATCTGCA-3′); nuclear factor κB (NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′); and GATA-2 (5′-CACTTGATAACAGAAAGTGATAACTCT-3′) were end-labeled with [γ-^{32}P]ATP by using the 5′-end-labeling kit from Amersham Pharmacia Biotech (Freiburg, Germany). Typically the binding mixture contained 3 to 10 μg of nuclear extract, 10,000 to 20,000 cpm of the ^{32}P-labeled oligonucleotide probe (0.5 ng), 1 μg of poly[*d*(I-C)], and 1.33 mM D,L-dithiothreitol in a total volume of 15 μl of binding buffer. After 30 min at room temperature, the DNA-protein complexes were resolved by nondenaturing 4% polyacrylamide gel electrophoresis. The gel was dried and the ^{32}P-labeled protein-DNA

complexes visualized by autoradiography. The specificity of the binding reaction was monitored by performing the assay in parallel with the same samples in the presence of a 100- to 1000-fold excess of the nonlabeled oligonucleotide. For supershift analyses, 1.0 to 2.0 μl of the appropriate gel supershift antibody (200 μg/0.1 ml, Santa Cruz Biotechnology) per 6.0 to 7.0 μl of nuclear extract (3–10 μg of protein) were preincubated overnight at 4°C or at room temperature for 60 min before the EMSA was performed.

Reporter Gene Analysis. The pAP-1(PMA)-TA-Luc vector (CLONTECH, Heidelberg, Germany) was used to show the activation of the transcription factor AP-1 by PDTC. The pCMV TK luc+ expression vectors (GenBank accession no. AF027129) containing the −168-bp fragment of the rat preproendothelin-1 promoter as well as the control vectors with and without the CMV promoter were prepared as described (Paul et al., 1995). Cotransfections for normalization of transfection efficacy were performed with the SV40/β-galactosidase expression vector pUC19 (GenBank accession no. M77789). For transfection, 50% confluent rat SMC were incubated with 1.5 μg of plasmid DNA and 18 μl of Effectene (Qiagen, Hilden, Germany) for 6 h; thereafter the medium was replaced and the cells cultured until they attained 80% confluence (usually after 18–24 h). They were then incubated with 10 to 100 μM PDTC or 0.1 μM phorbol 12-myristate 13-acetate (PMA) for 6 h. Luciferase and β-galactosidase activities in the cell lysates were determined by using corresponding chemiluminescence and photometric assay kits (Promega, Mannheim, Germany).

Decoy Oligodeoxynucleotide Technique. Double-stranded ODN were prepared from complementary single-stranded phosphorothioate-bonded ODN obtained from Eurogentec (Köln, Germany) by melting at 95°C for 5 min followed by a cool-down phase of 3 to 4 h at ambient temperature. The efficiency of the hybridization reaction was verified with 2.5% agarose gel electrophoresis and usually found to exceed 85%. The sequences of the single-stranded ODN were as follows (underlined letters denote phosphorothioate-bonded bases): AP-1, CGCTTGATGACTCAGCCGGAA; AP-1mut, CGCTTGATTACTTAGCCGGAA; C/EBP, TGCAGATTGCGCAATCTGCA; C/EBPmut, TGCAGAGACTAGTCTCTGCA; NF-κB, AGTTGAGGGGACTTTCCCAGGC; GATA-2, CACTTGATAACA-GAAAGTGATAACTCT. On the basis of previous EMSA and RT-PCR analyses, the maximally effective concentration and the optimal preincubation time for all decoy ODN in the cultured SMC were determined to be 10 μM and 4 h. Transfection of the decoy ODN was achieved without using any cationic lipid or liposomal complex. Thereafter the decoy ODN-containing medium was removed, cells were washed twice with medium, and then incubated in fresh medium containing the various test compounds for the indicated periods of time. Decoy ODN treatment also preceded transient transfection of the cultured SMC with the aforementioned reporter gene constructs.

Staining of Nuclear DNA with H 33342. After termination of the incubation period, SMC were washed twice with HBSS and then incubated with fixation buffer (5% formaldehyde in 145 mM NaCl, 10 mM Hepes × KOH, pH 7.5) for 20 min at room temperature. After this period the fluorescent bisbenzimide dye H 33342 (Calbiochem, Bad Soden, Germany) was added at a final concentration of 10 μg/ml and the cells were incubated for a second period of 20 min. Thereafter, the buffer was replaced by 50% glycerol in HBSS, and the fixed cells were examined by using a video imaging system (Visitron, München, Germany). Nuclear staining intensity and morphology were evaluated optically and documented by using the MetaMorph V3.0 software package (Universal Imaging Co., West Chester, PA).

Data Analysis. Unless indicated otherwise, results are expressed as mean ± S.E.M. of n observations. Student's unpaired t test was used to determine differences between the means with a P value <0.05 considered to be statistically significant.

Results

ET_B-R Expression. In aortic and mesenteric SMC grown on either plain plastic, fibronectin, gelatin, or collagen, basal ET_B-R mRNA levels were either not (Fig. 1, a and b) or only hardly detectable (Fig. 1c). Tumor necrosis factor α (TNFα; 1000 U/ml), interleukin-1β (IL-1β; 60 U/ml) or interferon-γ (IFNγ; 200 U/ml) alone or in combination (Fig. 1a) failed to induce ET_B-R expression. This was also the case when the aortic SMC had been isolated enzymatically instead of using the explant technique (not shown). The cytokines, on the other hand, especially when combined, significantly enhanced the expression of other gene products, such as iNOS and VCAM-1 mRNA (Fig. 2), in the cultured SMC. Moreover, in endothelium-intact or denuded ring segments of the rat aorta (i.e., native endothelial and smooth muscle cells), there was also no increase in ET_B-R mRNA abundance following 6 h of exposure to the cytokines (not shown).

Unexpectedly, PDTC, an inhibitor of NF-κB activation

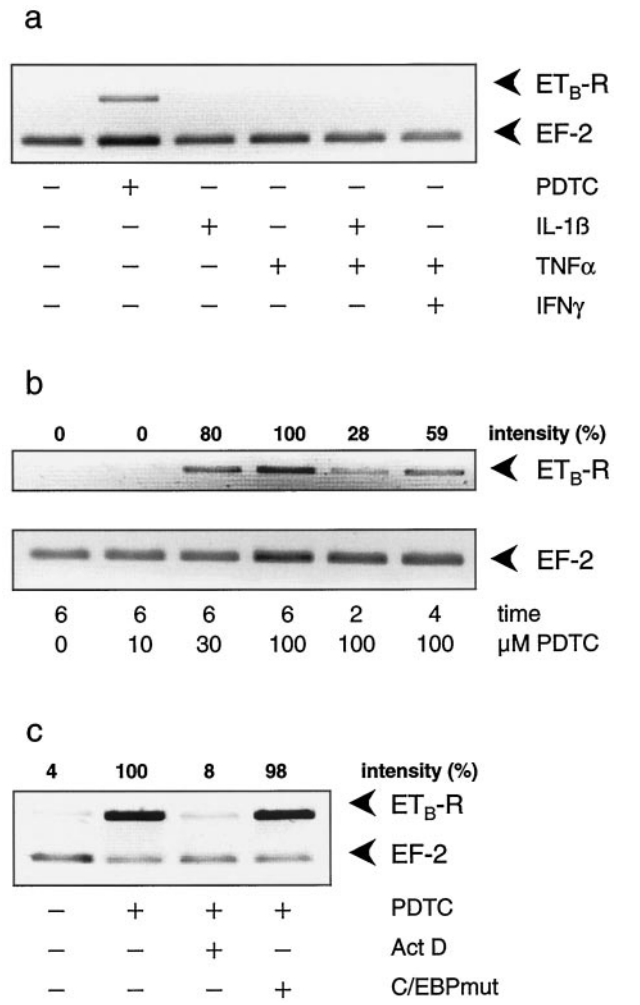

Fig. 1. a, ET_B-R expression in 100 μM PDTC-, 60 U/ml IL-1β-, 1000 U/ml TNFα-, or 200 U/ml interferon-γ-stimulated rat aortic cultured SMC (raSMC). The figure depicts a typical RT-PCR analysis; comparable findings were obtained in three further experiments with different batches of raSMC. b, time- and concentration-dependent effect of PDTC on ET_B-R expression in raSMC. The figure depicts a typical RT-PCR analysis with the relative intensities (%), as judged by densitometry, indicated at the top. Comparable findings were obtained in two further experiments with different batches of raSMC. c, effect of actinomycin D (Act D, 1 μM) and of the C/EBP mutant decoy ODN (C/EBPmut; 10 μM, 4-h preincubation) on PDTC-stimulated ET_B-R expression. The figure depicts a typical RT-PCR analysis with the relative intensities (%), as judged by densitometry, indicated at the top. Comparable findings were obtained in two further experiments with different batches of raSMC.

(Schreck et al., 1992) that was used to modulate cytokine-stimulated iNOS gene expression, elicited a pronounced increase in ET_B-R mRNA both in aortic and mesenteric SMC in a time- and concentration-dependent manner (maximum at 100 μM and 6 h; Fig. 1b). The cytokine-stimulated increase in iNOS and VCAM-1 mRNA abundance, on the other hand, was significantly inhibited in the presence of 100 μM PDTC (by 96 and 70%, respectively), whereas basal ET_A-R mRNA levels remained unchanged in PDTC-treated SMC (118 ± 11% of control, $n = 4$). Coincubation with actinomycin D (1 μM) abolished the PDTC-induced increase in ET_B-R mRNA (Fig. 1c). Moreover, PDTC-induced ET_B-R expression was reduced by approximately 30% in the presence of TNFα plus IL-1β (1 h of preincubation with PDTC before addition of the cytokines; Fig. 3). No difference could be detected in the ability of PDTC to stimulate ET_B-R expression in either type of SMC or in the native SMC. Moreover, the protein kinase C inhibitor, RO 31-8220 (0.1 μM), had no effect on PDTC-stimulated ET_B-R expression (not shown).

Transcription Factor Activation. EMSA of nuclear extracts prepared from the cultured SMC after 90 min of exposure to 100 μM PDTC alone revealed a significant increase in the amount of AP-1, two members of the C/EBP family of transcription factors, C/EBPβ and -δ, and of GATA-2 in the nucleus (Fig. 4). Addition of PDTC to the SMC 1 h before IL-1β and TNFα only slightly attenuated the cytokine-stimulated translocation of NF-κB to the nucleus, whereas the activity of both AP-1 and C/EBP was enhanced in response to this agent. Interestingly, PDTC at this concentration almost completely inhibited NF-κB activation in the presence of TNFα plus IFNγ (Krzesz et al., 1999). Cytokine-stimulated GATA-2 transfer to the nucleus, on the other hand, was modestly attenuated in the presence of PDTC (Fig. 4). Supershift analysis (Fig. 5) revealed two GATA-2-specific DNA-protein complexes in the nuclear extracts of the SMC. Further supershift analyses confirmed that PDTC enhanced mostly C/EBPβ (Fig. 5), and that the two NF-κB-specific complexes consisted of the p65/50 heterodimer and the p50/50 homodimer (Krzesz et al., 1999).

Effects of the Decoy ODN. Pretreatment of the cultured SMC with the AP-1, C/EBP, or GATA-2-specific decoy ODN individually reduced PDTC-induced ET_B-R expression to 51, 47, and 66% of the stimulated control, respectively (Fig. 3).

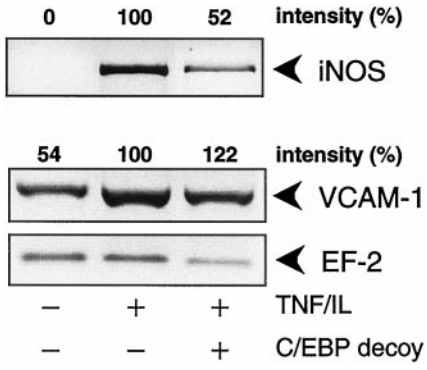

Fig. 2. Effect of 10 μM C/EBP consensus decoy ODN (4-h preincubation) on 1000 U/ml TNFα plus 60 U/ml IL-1β- stimulated (TNF/IL) iNOS and VCAM-1 expression. The figure depicts a typical RT-PCR analysis with the relative intensities (%), as judged by densitometry, indicated at the top. Comparable findings were obtained in two further experiments with different batches of raSMC.

The NF-κB-specific decoy ODN (Fig. 3b) and the AP-1 and C/EBP mutant decoy ODN, which were used as controls, on the other hand, had no effect on PDTC-induced ET_B-R expression (Fig. 1c). According to EMSA, the decoy ODNs were highly specific for their target transcription factor. Thus, abundance of the target transcription factor was significantly reduced in nuclear extracts prepared from cells treated with the corresponding decoy ODN, whereas translocation of other transcription factors to the nucleus was not affected (Fig. 6). Moreover, pretreatment with the C/EBP consensus decoy ODN significantly reduced cytokine-stimulated iNOS gene expression, which is thought to be C/EBP-dependent (Eberhardt et al., 1998), in the aortic SMC; however, VCAM-1 gene expression, which is thought to be predominantly NF-κB-dependent (Libby et al., 1995), remained largely unaffected (Fig. 2).

Further proof for the specificity of the decoy ODN approach was obtained in experiments in which the cultured SMC were transiently transfected with a truncated (168 bp) rat preproendothelin-1 promoter construct containing a single, but functionally crucial (Lauth et al., 2000b), AP-1 binding site. The AP-1 consensus decoy ODN, but not the corresponding mutant decoy ODN, markedly reduced both basal and phorbol ester or PDTC-stimulated luciferase activity in these cells (Fig. 7). These effects of the AP-1 decoy ODN were more

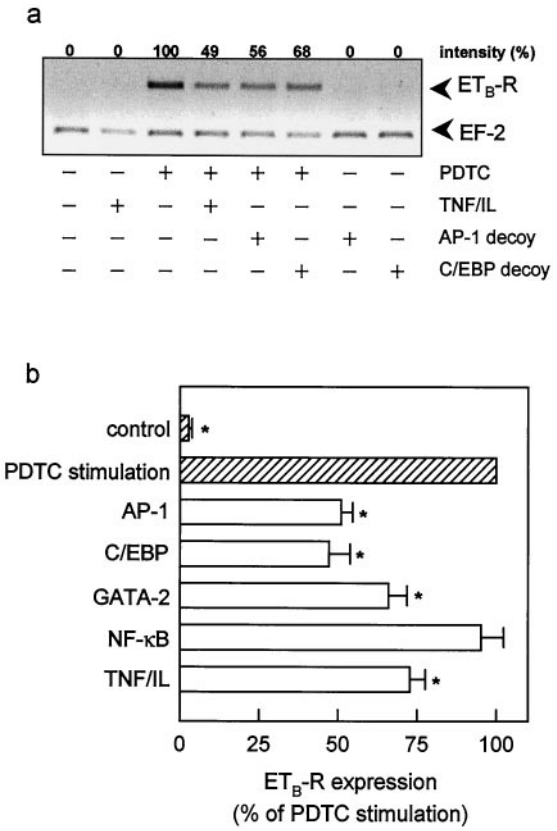

Fig. 3. a, effect of TNF/IL and 10 μM each of the AP-1 and C/EBP consensus decoy ODN (4-h preincubation) on 100 μM PDTC-stimulated ET_B-R expression in rat mesenteric cultured SMC incubated for 6 h. The figure depicts a typical RT-PCR analysis with the relative intensities (%), as judged by densitometry, indicated at the top. b, statistical summary of the effects of the consensus AP-1, C/EBP, and GATA-2-specific decoy ODN on PDTC-stimulated ET_B-R expression (calculated as percentage of the stimulated control) in rat aortic and rat mesenteric cultured SMC ($n = 3$–7; $^*P < .05$ versus PDTC).

apparent in SMC grown in serum-reduced medium (0.5% FBS) as compared with complete medium (10% FBS), suggesting that the reporter gene construct also responded to serum, thus reducing the efficacy of the decoy ODN (not shown). The fact that the mutant decoy ODN also displayed a weak inhibitory effect can be attributed to the relatively high concentration used in these experiments, which, according to subsequent EMSA, renders this slightly modified decoy ODN (as compared with the consensus AP-1 decoy ODN, see *Materials and Methods*) effective to some extent in scavenging AP-1 (not shown). One additional conclusion that could be drawn from these reporter gene analyses was that PDTC also functionally activated AP-1, and this was verified by its concentration-dependent effects on the expression of a synthetic AP-1 reporter gene transiently transfected into the cultured SMC (Fig. 7a, insert).

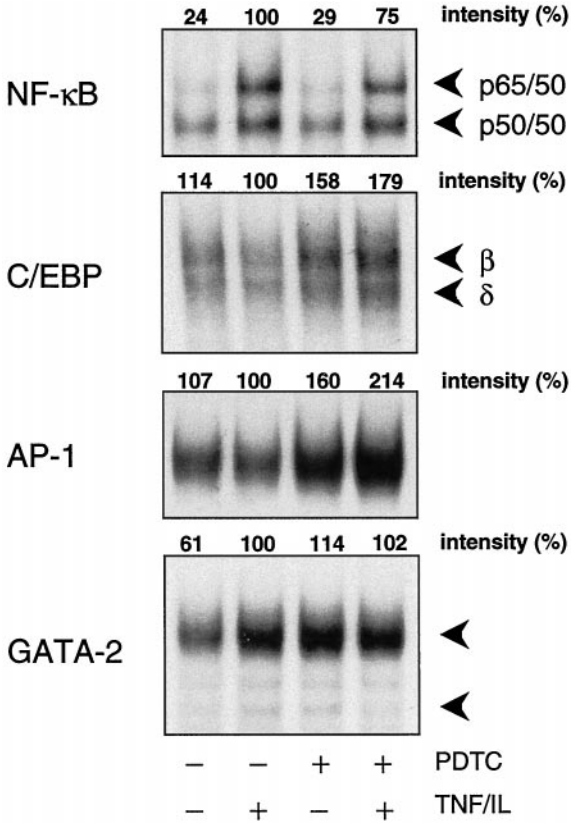

Fig. 4. Effect of PDTC (100 μM) on basal- and TNF/IL-stimulated nuclear translocation of NF-κB (p65/p50 heterodimer and p50/p50 homodimer), C/EBP (β and δ isoforms), AP-1, and GATA-2 (two complexes as indicated by the arrows) in rat aortic cultured SMC. The figure depicts a typical EMSA with the relative intensities (%), as judged by densitometry, indicated at the top. Qualitatively identical results were obtained with at least two further batches of SMC for each transcription factor.

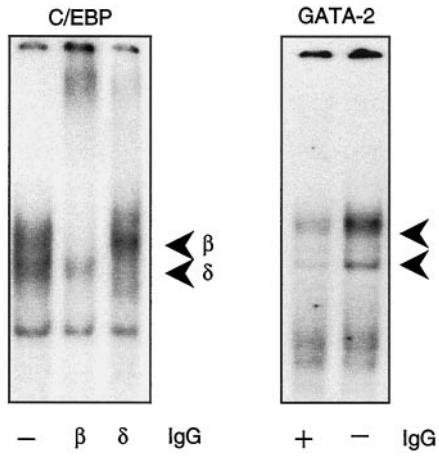

Fig. 5. Identification of C/EBP (β and δ isoforms) and GATA-2 (two complexes as indicated by the arrows) in nuclear extracts of rat aortic cultured SMC. The figure depicts typical EMSA with the appropriate supershift analysis performed with a single batch of SMC. Identical results were obtained with another batch of raSMC for each transcription factor.

PDTC-Induced Expression of Functional ET_B-R Protein. To verify that the PDTC-induced rise in ET_B-R mRNA abundance was accompanied by a corresponding increase in receptor protein, Western blot analyses with an ET_B-*R*-specific polyclonal antibody were performed. A protein band of the expected molecular mass (43 kDa, GenBank accession no. 111606) was detected that was up-regulated in PDTC-treated cultured SMC to a variable extent (Fig. 8). Pretreatment of the cells with the AP-1 consensus decoy ODN, but not with the corresponding mutant decoy ODN, completely abrogated this PDTC-induced increase in ET_B-R protein (Fig. 8). In addition, the presence of functional ET_B-R protein was investigated by monitoring ET-1-induced apoptosis of the SMC. The reason for choosing this bioassay method was our recent observation of an enhanced rate of apoptosis in rat aortic cultured SMC in the presence of exogenous ET-1 when ET_B-R expression had been up-regulated by exposing the cells to cyclic strain (Cattaruzza et al., 2000). Previously, ET_B-R-mediated apoptosis was verified by flow cytometry, chromatin condensation, DNA ladder formation, and caspase-3 activation; for the present study we analyzed chromatin condensation only.

Neither ET-1 nor BQ 788 alone had a significant effect on SMC apoptosis, whereas PDTC alone caused an approximately 3-fold increase in the number of apoptotic nuclei (Fig. 9), which, however, did not attain statistical significance. On the other hand, PDTC in concentrations up to 100 μM did not exert a cytotoxic effect, as judged by light microscopy and the MTT assay (not shown). In the presence of both PDTC and ET-1, SMC apoptosis increased more than 8-fold, and this pro-apoptotic effect was reduced to the level of PDTC alone in the presence of BQ 788, hence confirming the PDTC-induced increase in ET_B-R protein at the functional level.

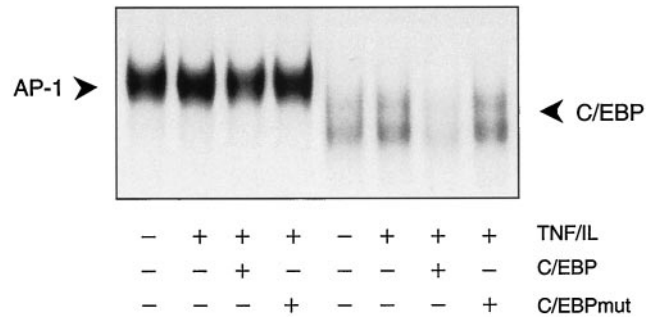

Fig. 6. Example of the specificity of the consensus decoy ODN for C/EBP (Fig. 2). The abundance of the target transcription factor is significantly reduced in nuclear extracts from consensus but not mutant decoy ODN-treated rat aortic cultured SMC, whereas that of AP-1 is not affected. The figure depicts a typical EMSA. Qualitatively identical results were obtained with a least four further batches of raSMC for each transcription factor.

Discussion

The present findings demonstrate that in contrast to other cytokine-inducible gene products (CD40, iNOS, monocyte chemoattractant protein-1, VCAM-1) ET_B-R expression in rat cultured SMC is not up-regulated in the presence of IFNγ, IL-1β, TNFα, or their combination. It was shown recently that ET_B-R expression in human umbilical vein endothelial cells is significantly increased by TNFα when the cells are grown on a fibrin matrix, but down-regulated in cells cultured on plain plastic (Smith et al., 1998). In the present study with SMC grown on plastic, collagen, fibronectin, or gelatin-coated culture dishes we observed either no effect on basal ET_B-R expression or a decrease in PDTC-stimulated ET_B-R mRNA expression by this and the two other cytokines.

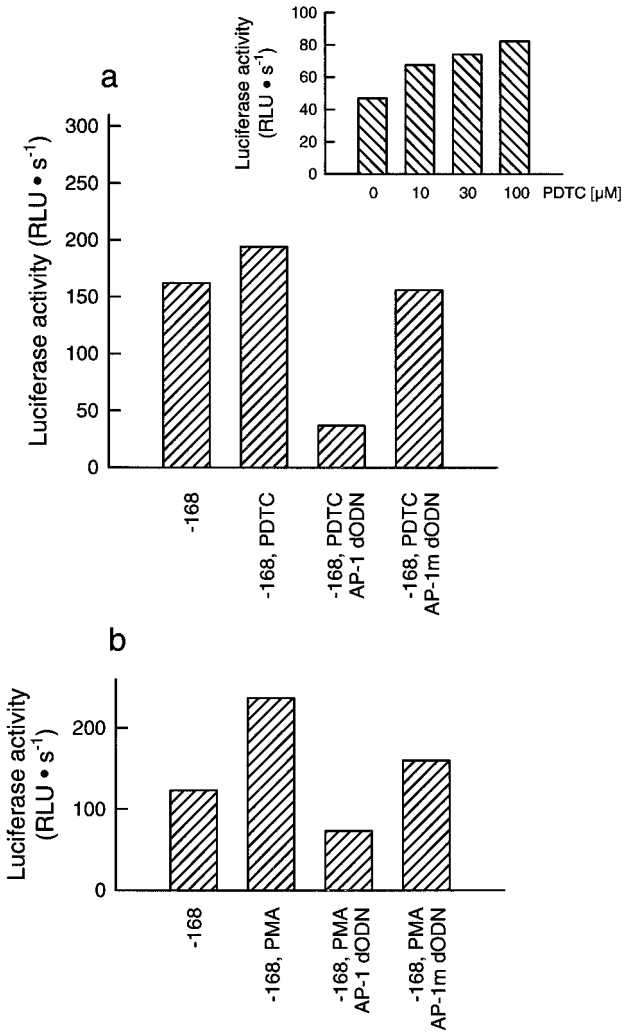

Fig. 7. Expression of the −168-bp fragment of the rat preproendothelin-1 promoter construct in rat aortic cultured SMC incubated for 6 h with 100 μM PDTC (a) or 0.1 μM phorbol ester (PMA) (b) with or without 10 μM AP-1 or AP-1mut decoy ODN (dODN denotes the decoy ODN with the mutated core binding site for the AP-1 transcription factor). Qualitatively identical results were obtained with at least two further batches of SMC for each stimulus. Basal luciferase activity in the presence of the AP-1 decoy ODN alone was reduced to 55 ± 14% of control (n = 6). a, the insert shows that PDTC also functionally activates AP-1 verified by its concentration-dependent effects on the expression of a synthetic AP-1 reporter gene transiently transfected into the cultured SMC. Reporter gene, i.e. luciferase activity values were normalized with β-galactosidase activities in the cell lysates.

Moreover, there was no cytokine-inducible ET_B-R expression in native endothelial and smooth muscle cells in situ (rat aorta); thus, these cytokines are unlikely to play a role in ET_B-R induction in the rat vasculature.

The pyrrolidine derivative of dithiocarbamate, PDTC, is widely used as an inhibitor of NF-κB activation in intact cells (Schreck et al., 1992). It is used as a tool to explore the expression of genes involved in inflammatory processes, and an inhibitory effect of PDTC on gene expression is usually linked exclusively to a prevention of NF-κB activation. The present data clearly show that PDTC does not only affect the activity of NF-κB but also that of other transcription factors such as AP-1, C/EBP, and GATA-2. Moreover, recent findings from our laboratory also demonstrate that PDTC inhibits the IFNγ-stimulated activation of Stat-1 in rat aortic cultured SMC (Krzesz et al., 1999). An activation by PDTC of AP-1 leading to an increased expression of other gene products

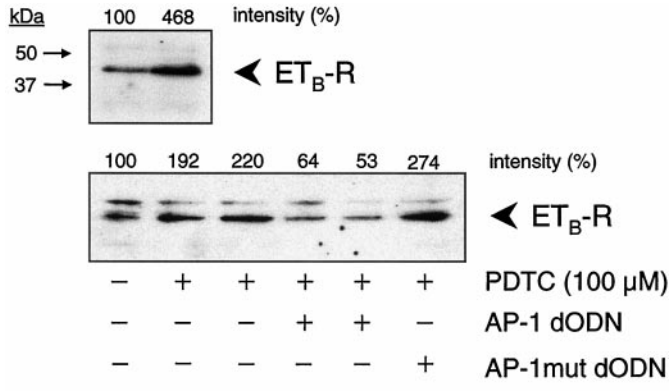

Fig. 8. Effect of PDTC (100 μM) on ET_B-R protein expression in rat aortic cultured SMC in the presence or absence of 10 μM AP-1 consensus or mutant (mut) decoy ODN (dODN). The figure depicts two typical Western blots with the relative intensities (%), as judged by densitometry, indicated at the top. Qualitatively identical results were obtained with at least two further batches of raSMC.

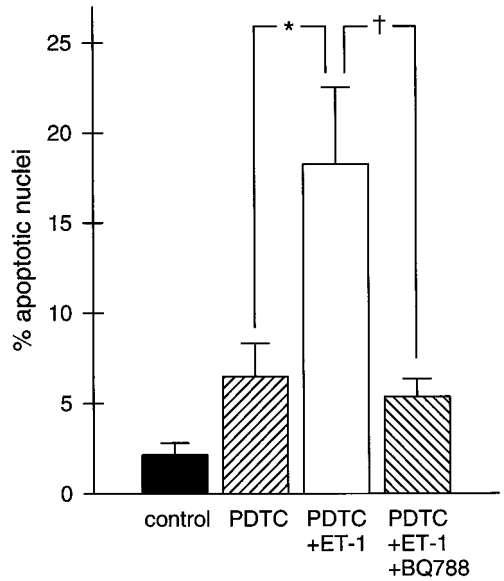

Fig. 9. Percentage of apoptosis in rat aortic cultured SMC exposed to PDTC (100 μM) alone, PDTC plus ET-1 (10 nM), or PDTC plus ET-1 plus BQ788 (1 μM) for 18 h. Nuclei were stained with H 33342 as indicated under *Materials and Methods* (n = 4–6; *, $P < .05$ versus PDTC; †, $P < .05$ versus PDTC plus ET-1).

such as heme oxygenase-1 (Hartsfield et al., 1998), manganese superoxide dismutase (Borrello and Demple, 1997), or intracellular adhesion molecule-1 (Munoz et al., 1996) has also been reported. These effects and that on ET_B-R expression, however, may not be related to PDTC itself, but to thiuramdisulfides, the reactive metabolites of these dithiocarbamates (Liu et al., 1998).

The RNA synthesis inhibitor, actinomycin D, completely abolished PDTC-stimulated ET_B-R expression, suggesting that PDTC increased the rate of transcription of the ET_B-R gene. To elucidate the transcriptional mechanism by which PDTC up-regulated ET_B-R expression in the SMC, four transcription factors (i.e., AP-1, C/EBP, NF-κB, and GATA-2) that we considered to be the most promising candidates were investigated. AP-1 was the prime candidate because its activation by PDTC had been described before (see above). In addition, preproET-1 gene expression is regulated by transactivating signals that depend on a cooperative interaction of GATA-2 and AP-1 *cis*-elements (Kawana et al., 1995). We reasoned that if expression of the endothelin system, as in the rat carotid artery following PTCA (Wang et al., 1996), is mediated by the same transcription factors, then GATA-2 should also be a prime candidate. Moreover, from parallel work in our laboratory on deformation-induced expression of the ET_B-R in rabbit blood vessels (Lauth et al., 2000a) C/EBPβ or -δ had emerged as another important transcription factor. As judged by EMSA and corresponding supershift analyses, PDTC indeed increased the abundance of C/EBP (in particular that of C/EBPβ), AP-1, and GATA-2 in nuclear extracts of the cultured SMC. Both basal- and TNFα plus IL-1β-stimulated NF-κB activity, on the other hand, were only moderately affected by PDTC, whereas NF-κB activation by TNFα plus IFNγ was strongly reduced (Krzesz et al., 1999).

Because the sequence of the 5′-flanking region of the rat ET_B-R gene is as yet unknown, we used consensus decoy ODN to verify that the aforementioned three transcription factors are responsible for the PDTC-induced up-regulation of ET_B-R transcription. Phosphorothiorate-modified oligonucleotides are fully resistant to almost all nucleases and have a high biological stability (Monia et al., 1996). Only after thoroughly testing for their target specificity and optimization of both preincubation time and concentration by EMSA were the decoy ODN used. PDTC-stimulated ET_B-R expression in the cultured SMC was indeed significantly decreased, albeit to a different degree after exposure to the consensus decoy ODN against AP-1, C/EBP, or GATA-2, but not when the corresponding mutant decoy ODN were used. The NF-κB-specific decoy ODN, on the other hand, had no effect on PDTC-induced ET_B-R expression. The specificity of the decoy ODN technique was further substantiated by reporter gene analysis with an AP-1-driven preproendothelin-1 promoter luciferase gene construct transiently transfected into the cultured SMC. These reporter gene analyses also confirmed the PDTC-induced increase in AP-1 activity at the functional level.

On the basis of these findings we conclude that the enhanced transcription of the ET_B-R gene in rat cultured SMC in the presence of PDTC is mediated by a PDTC-induced increase in the activity of AP-1, C/EBPβ, and GATA-2. This conclusion is indirectly supported by the putative binding sites for transcription factors that can be identified in the 5′-flanking region of the human ET_B-R gene (GenBank accession no. D13162), with the reservation that there is indeed a sufficient homology between rat and human ET_B-R genes in the promoter region. By using the MatInspector V2.2 software package (Quandt et al., 1995), we detected a potential binding site for GATA at −995 bp; three C/EBP sites at −437, −348, and −59 bp; and four AP-1 sites at −706, −685, −369, and −201 bp. The 5′-flanking region of the human ET_B-R gene does not seem to contain a classical TATA box or binding sites for NF-κB when scanned with the appropriate stringency. The IL-1β/TNFα-mediated modest decrease in PDTC-stimulated ET_B-R expression, which could be explained by the observed decrease in GATA-2 activity, also argues in favor of an involvement of this transcription factor in ET_B-R gene expression.

Exposure of the cultured SMC to PDTC also resulted in an increase in functional ET_B-R protein, as verified by the BQ 788-sensitive, ET-1-induced increase in apoptosis. Such a pro-apoptotic effect can also be observed in rat aortic cultured SMC exposed to cyclic strain that is based on an activation of the ET_B-R but not the ET_A-R (Cattaruzza et al., 2000), which predominates in these cells under static conditions. One functional consequence of this ET_B-R-mediated apoptosis, which can also be observed in native arterial but not venous SMC in situ (Lauth et al., 2000a), may be the remodeling of the vessel wall in response to a pressure overload such as in restenosis following PTCA (Bennett, 1999) or arterial hypertension. Knowing the transcription factors that mediate this pressure-induced increase in ET_B-R expression in the SMC may help to devise new strategies by which this possibly deleterious adaptive response can be prevented.

In summary, the present findings demonstrate that in cultured SMC of the aorta and mesenteric vascular bed of the rat, ET_B-R gene expression is up-regulated by PDTC. This stimulation of ET_B-R gene expression appears to be mediated by the simultaneous activation of AP-1, C/EBPβ, and GATA-2. Although it is not clear how PDTC up-regulates transcription factor activity, presumably by influencing the activity of certain protein kinases and/or phosphatases, this effect may not be caused by PDTC itself, but by the corresponding thiuramdisulfide metabolite. Regardless of the precise mechanism of action of PDTC, this agent may be used as a surrogate stimulus for the effects of cyclic strain on gene expression in cultured SMC that also seems to be associated with activation of a similar set of transcription factors. The highly significant suppression of PDTC-stimulated ET_B-R expression by the corresponding decoy ODN finally verifies the feasibility of the decoy ODN strategy for studying the transcriptional regulation of disease-related genes in vitro and may contribute to the development of new therapeutic concepts on this basis.

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References

Bennett MR (1999) Apoptosis of vascular smooth muscle cells in vascular remodelling and atherosclerotic plaque rupture. *Cardiovasc Res* **41:**361–368.

Borrello S and Demple B (1997) NF κB-independent transcriptional induction of the human manganous superoxide dismutase gene. *Arch Biochem Biophys* **348:**289–294.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram

quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.
Cattaruzza M, Dimigen C, Ehrenreich H and Hecker M (2000) Stretch-induced endothelin B receptor-mediated apoptosis in vascular smooth muscle cells. *FASEB J* **14:**991–998.
Cramer H, Muller-Esterl W and Schroeder C (1997) Subtype-specific desensitization of human endothelin ETA and ETB receptors reflects differential receptor phosphorylation. *Biochemistry* **36:**13325–13332.
Dashwood MR, Mehta D, Izzat MB, Timm M, Bryan AJ, Angelini GD and Jeremy JY (1998) Distribution of endothelin-1 (ET) receptors (ET_A and ET_B) and immunoreactive ET-1 in porcine saphenous vein-carotid artery interposition grafts. *Atherosclerosis* **137:**233–242.
Denizot F and Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* **89:**271–277.
Douglas SA and Ohlstein EH (1997) Signal transduction mechanisms mediating the vascular actions of endothelin. *J Vasc Res* **34:**152–164.
Douglas SA, Vickery-Clark LM, Louden C and Ohlstein EH (1995) Selective ET_A receptor antagonism with BQ-123 is insufficient to inhibit angioplasty induced neointima formation in the rat. *Cardiovasc Res* **29:**641–646.
Eberhardt W, Pluss C, Hummel R and Pfeilschifter J (1998) Molecular mechanisms of inducible nitric oxide synthase gene expression by IL-1β and cAMP in rat mesangial cells. *J Immunol* **160:**4961–4969.
Etore F, Tenu JP, Teiger E, Adnot S, Lonchampt MO, Pirotzki E and Le Doan T (1998) Sequence dependency of the internalization and distribution of phosphorothioate oligonucleotides in vascular smooth muscle cells. *Biochem Pharmacol* **55:**1465–1473.
Hartsfield CL, Alam J and Choi AM (1998) Transcriptional regulation of the heme oxygenase 1 gene by pyrrolidine dithiocarbamate. *FASEB J* **12:**1675–1682.
Hecker M, Mulsch A, Bassenge E, Forstermann U and Busse R (1994) Subcellular localization and characterization of nitric oxide synthase(s) in endothelial cells: Physiological implications. *Biochem J* **299:**247–252.
Kawana M, Lee ME, Quertermous EE and Quertermous T (1995) Cooperative interaction of GATA-2 and AP1 regulates transcription of the endothelin-1 gene. *Mol Cell Biol* **15:**4225–4231.
Kirchengast M and Munter K (1999) Endothelin-1 and endothelin receptor antagonists in cardiovascular remodeling. *Proc Soc Exp Biol Med* **221:**312–325.
Krzesz R, Wagner AH, Cattaruzza M and Hecker M (1999) Cytokine-inducible CD40 gene expression in vascular smooth muscle cells is mediated by nuclear factor κB and signal transducer and activation of transcription-1. *FEBS Lett* **453:**191–196.
Lauth M, Berger MM, Cattaruzza M and Hecker M (2000a) Elevated perfusion pressure upregulates endothelin-1 and endothelin B receptor expression in the rabbit carotid artery. *Hypertension* **35:**648–654.
Lauth M, Wagner AH, Cattaruzza M, Orzechowski HD, Paul M and Hecker M (2000b) Transcriptional control of deformation-induced preproendothelin-1 gene expression. *J Mol Med,* in press.
Libby P, Sukhova G, Lee RT and Galis ZS (1995) Cytokines regulate vascular functions related to stability of the atherosclerotic plaque. *J Cardiovasc Pharmacol* **25 (Suppl 2):**S9–S12.
Liu GY, Frank N, Bartsch H and Lin JK (1998) Induction of apoptosis by thiuram-disulfides, the reactive metabolites of dithiocarbamates, through coordinative modulation of NFkappaB, c-fos/c-jun, and p53 proteins. *Mol Carcinogen* **22:**235–246.
Monia BP, Johnston JF, Sasmor H and Cummins LL (1996) Nuclease resistance and antisense activity of modified oligonucleotides targeted to Ha-ras. *J Biol Chem* **271:**14533–14540.
Morishita R, Higaki J, Tomita N and Ogihara T (1998) Application of transcription factor "decoy" strategy as means of gene therapy and study of gene expression in cardiovascular disease. *Circ Res* **82:**1023–1028.
Munoz C, Castellanos MC, Alfranca A, Vara A, Esteban MA, Redondo JM and de Landazuri MO (1996) Transcriptional up-regulation of intracellular adhesion molecule-1 in human endothelial cells by the antioxidant pyrrolidine dithiocarbamate involves the activation of activating protein-1. *J Immunol* **157:**3587–3597.
Parris RJ and Webb DJ (1997) The endothelin system in cardiovascular physiology and pathophysiology. *Vasc Med* **2:**31–43.
Paul M, Zintz M, Böcker and Dyer JS (1995) Characterization and functional analysis of the rat endothelin-1 promoter. *Hypertension* **25:**683–693.
Phillips MI and Gyurko R (1997) Antisense oligonucleotides: New tools for physiology. *News Physiol Sci* **12:**99–105.
Porter KE, Olojugba DH, Masood I, Pemberton M, Bell PR and London NJ (1998) Endothelin-B receptors mediate intimal hyperplasia in an organ culture of human saphenous vein. *J Vasc Surg* **28:**695–701.
Quandt K, Frech K, Karas H, Wingender E and Werner T (1995) MatInd and MatInspector: New fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* **23:**4878–4884.
Schiffrin EL, Intengan HD, Thibault G and Touyz RM (1997) Clinical significance of endothelin in cardiovascular disease. *Curr Opin Cardiol* **12:**354–367.
Schreck R, Meier B, Mannel DN, Droge W and Baeuerle PA (1992) Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J Exp Med* **175:**1181–1194.
Smith PJ, Teichert-Kuliszewska K, Monge JC and Stewart DJ (1998) Regulation of endothelin-B receptor mRNA expression in human endothelial cells by cytokines and growth factors. *J Cardiovasc Pharmacol* **31 (Suppl 1):**S158–S160.
Tsujino M, Hirata Y, Eguchi S, Watanabe T, Chatani F and Marumo F (1995) Nonselective ET_A/ET_B receptor antogonist blocks proliferation of rat vascular smooth muscle cells after balloon angioplasty. *Life Sci* **56:**449–454.
Wang X, Douglas SA, Louden C, Vickery-Clark LM, Feuerstein GZ and Ohlstein EH (1996) Expression of endothelin-1, endothelin-3, endothelin-converting enzyme-1, and endothelin-A and endothelin-B receptor mRNA after angioplasty-induced neointimal formation in the rat. *Circ Res* **78:**322–328.
Yamashita J, Yoshimasa T, Arai H, Hiraoka J, Takaya K, Miyamoto Y, Ogawa Y, Itho H and Nakao K (1998) Identification of cis-elements of the human endothelin-A receptor gene and inhibition of the gene expression by the decoy strategy. *J Biol Chem* **273:**15993–15999.

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